# Mechanistic Studies of Ionizing Radiation and Oxidative Mutagenesis: Genetic Effects of a Single 8-Hydroxyguanine (7-Hydro-8-oxoguanine) Residue Inserted at a Unique Site in a Viral Genome†

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ABSTRACT: T4 RNA ligase was used to construct a deoxypentanucleotide containing a single 8-hydroxyguanine (7-hydro-8-oxoguanine; $G^{8-OH}$) residue, which is one of the putatively mutagenic DNA adducts produced by oxidants and ionizing radiation. The pentamer d(GCTAG$^{8-OH}$)p was prepared by the ligation of a chemically synthesized acceptor molecule, d(GCTA), to an adducted donor, 8-hydroxy-2′-deoxyguanosine 5′,3′-bisphosphate. The acceptor was efficiently converted to the reaction product (>95%), and the final product yield was 50%. Following 3′-dephosphorylation, the pentamer was characterized by UV spectroscopy, by high-pressure liquid chromatography, and by gas chromatography–mass spectrometry of the nucleosides released by enzymatic hydrolysis. Both d(GCTAG$^{8-OH}$) and an unmodified control were 5′-phosphorylated by using [$\gamma$-$^{32}$P]ATP and incorporated covalently by DNA ligase into a five-base gap at a unique *Nhe*I restriction site in the otherwise duplex genome of an M13mp19 derivative. The ligation product contained $G^{8-OH}$ at the 3′ residue of an in-frame *amber* codon (5′-TAG-3′) (genome position 6276) of the phage *lacZ*$\alpha$ gene. The adduct was part of a nonsense codon in a unique restriction site in order to facilitate the identification and selection of mutants generated by the replication of the modified genome in *Escherichia coli*. Both control and adducted pentamers ligated into the genome at 50% of the maximum theoretical efficiency, and nearly all (~90%) of the site-specifically adducted products possessed pentanucleotides that were covalently linked at both 5′ and 3′ termini. The $G^{8-OH}$ lesion in the *Nhe*I site inhibited the cleavage of the site by a 200-fold excess of *Nhe*I. Transformation of *E. coli* strain DL7 with the uniquely modified single-stranded genome resulted in ~0.5–1.0% of the progeny phage showing the G → T transversion mutation at the original position of $G^{8-OH}$; no such mutations were observed from control genomes containing guanine in place of $G^{8-OH}$. The vector containing $G^{8-OH}$ also transformed 50–90% as efficiently as the unmodified control, indicating that the adduct can be both weakly cytotoxic and mutagenic to the phage genome.

Ionizing radiation and chemical oxidants can be toxic, mutagenic, and carcinogenic. The major cause of these effects is probably macromolecular damage, mainly mediated in aerobic systems by hydroxyl (OH•)[1] radicals, which induce DNA base modifications, protein–DNA cross-links, DNA strand breaks, and apurinic/apyrimidinic (AP) sites (von Sonntag, 1987; Teoule, 1987; Teebor et al., 1988). Most studies have emphasized the interactions of radicals with DNA in view of the perceived central role of DNA damage in the initiation and possibly later stages of carcinogenesis.

Much research has focused on the types of DNA lesions formed following DNA irradiation or reaction with oxygen radicals (von Sonntag, 1987; Teoule, 1987; Teebor et al., 1988). This work has been structural in its emphasis, however, and there are few data presently available to indicate the genotoxic properties of specific DNA lesions formed in irradiated or oxidized DNA. Historically, the work in this field has concentrated on the pyrimidines, particularly thymine, as these bases are highly susceptible to oxygen radical attack. Oxidation of the 5,6-double bond of thymine in vivo leads to a collection of products, the major one being *cis*-5,6-thymine glycol (t′) isomers (Teoule et al., 1974; Cathcart et al., 1984). Evidence that t′ is deleterious to the cell is suggested by the presence of specific repair proteins for t′ in both prokaryotes and eukaryotes (Higgins et al., 1987). Thymine glycol adducts are generally strong blocks to DNA replication in vitro (Rouet & Essigmann, 1985; Ide et al., 1985; Clark & Beardsley, 1986; Hayes & LeClerc, 1986). Recently, it has been shown that a single t′ inserted into single-stranded M13mp19 DNA is mutagenic and induces T → C transitions after transfection of the vector into *Escherichia coli* (Basu et al., 1989).

There has been a recent upsurge of interest in the radiation chemistry of the purines (von Sonntag, 1987; Teoule, 1987; Teebor et al., 1988), largely owing to the demonstration that 8-hydroxyguanine ($G^{8-OH}$) is potently mutagenic in vitro (Kuchino et al., 1987). This adduct has been detected in DNA

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[1] Abbreviations: AP, apurinic/apyrimidinic; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $G^{8-OH}$, 8-hydroxyguanine; GC–MS, gas chromatography–mass spectrometry; GHD, gapped-heteroduplex DNA; d(GCTAG$^{8-OH}$), deoxypentanucleotide containing an $G^{8-OH}$ adduct at the 3′ terminus; HCC, hexaamminecobalt chloride; HPLC, high-pressure liquid chromatography; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; M13+7-(*Stu*I) and M13+12-(*Nhe*I), insertion mutants of M13mp19 containing the recognition sequences of *Stu*I and *Nhe*I, respectively; nt, nucleotide; OH•, hydroxyl radical; pdGp, 2′-deoxyguanosine 5′,3′-bisphosphate; pdG$^{8-OH}$p, 8-hydroxy-2′-deoxyguanosine 5′,3′-bisphosphate; PEG 8000, poly(ethylene glycol) 8000; RF, replicative form; t′, *cis*-5,6-thymine glycol; TMS, trimethylsilyl; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; X-Gal, 5-bromo-4-chloro-3-indolyl $\beta$-D-galactopyranoside.

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following exposure to a range of OH• producing sources either in vivo (Kasai et al., 1986, 1987) or in vitro (Kasai et al., 1984; Dizdaroglu, 1985; Floyd et al., 1986). Kasai et al. (1986) have also suggested that $G^{8-OH}$ can be repaired in vivo since a time-dependent decrease in the amount of $G^{8-OH}$ in the liver DNA of γ-irradiated mice was noted in the 90-min period immediately following exposure. It was the goal of the work described below to develop a system whereby the genetic effects of $G^{8-OH}$ could be investigated in vivo. The chief obstacle to obtaining this goal was the unavailability of a genome containing the $G^{8-OH}$ adduct in isolation from all other DNA modifications. This obstacle was overcome by the synthesis of an oligonucleotide containing a single $G^{8-OH}$, which subsequently was inserted into the genome of a bacterial virus.

## Experimental Procedures[2]

*Materials.* Chemicals and supports required for modified phosphotriester or phosphoramidite oligonucleotide synthesis were purchased from either Applied Biosystems or Cruachem. Oligonucleotide Purification Cartridges were from Applied Biosystems. 2′-Deoxyguanosine 5′,3′-bisphosphate (pdGp), Sephadex G-10, Sephadex-SP C-25, bacteriophage T4 polynucleotide kinase, and the initial stock of bacteriophage M13mp19 DNA were from Pharmacia. Bacteriophage T4 RNA ligase was obtained from both Pharmacia and Bethesda Research Laboratories, the latter also supplying bacterial alkaline phosphatase. Phosphocreatine, adenylate kinase (type V), creatine phosphokinase (type I), spermine, dithiothreitol (DTT), snake venom phosphodiesterase (type II), bovine serum albumin (acetylated), and Sepharose CL-4B were purchased from Sigma. DE-52 DEAE-cellulose was from Whatman. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), isopropyl β-D-thiogalactopyranoside (IPTG), ATP, and calf intestinal phosphatase were supplied by Boehringer Mannheim. T4 DNA ligase and restriction enzymes were from New England Biolabs, and [γ-$^{32}$P]ATP (6000 Ci/mmol) was purchased from New England Nuclear. All other chemicals and solvents were used at the highest purity commercially available, except for triethylamine, which was used after redistillation. The *E. coli* strains, GW5100 (*lac*Z, *sup*E) and MM294A (*lac*$^+$, *ung*$^+$), were gifts from G. Walker (Department of Biology, MIT, Cambridge, MA) and K. Backman (Biotechnica International, Cambridge, MA), respectively. *E. coli* strain DL7 is a *lac*$^-$ derivative of strain MM294A and was constructed in this laboratory (Lasko et al., 1988).

*General Methods.* Anion-exchange high-pressure liquid chromatography (HPLC) of oligonucleotides and nucleotide (nt) bisphosphates was conducted with a 25 × 0.46 cm Spherisorb 5-μm SAX column on a Waters system comprised of two M-45 pumps, a Model 440 UV absorbance detector (254 nm), and an automated gradient controller. Reversed-phase HPLC was performed with a Phenomenex Ultremex 5-μm column (25 × 0.46 cm) on a Beckman system fitted with twin 114M pumps and a 421A gradient controller. These were attached to a Hewlett Packard 1040A diode-array detection system set to monitor absorbance at 260 nm. Chemical synthesis of oligonucleotides d(GCTA) and d(GCTAG) was carried out on a Cruachem manual module as described by Basu et al. (1987). The products were deprotected in concentrated $NH_4OH$ at 60 °C overnight and purified by anion-exchange HPLC (0–0.3 M $KH_2PO_4$ over 30 min at a flow rate of 1.0 mL/min) followed by reversed-phase HPLC if necessary. The relative nucleoside contents of these oligonucleotides were determined by reversed-phase HPLC after digestion of ~0.25 $A_{260}$ units of the compounds with snake venom phosphodiesterase and bacterial alkaline phosphatase by the method of Fowler et al. (1982). The 47- and 52-base oligonucleotides required for preparation of M13 insertion mutants were synthesized by the phosphoramidite procedure on an Applied Biosystems 381A automated DNA synthesizer and purified through Oligonucleotide Purification Cartridges.

Gas chromatography–mass spectrometry (GC–MS) was performed on a Model 5970B mass-selective detector interfaced to a Model 5890A gas chromatograph (both from Hewlett Packard) equipped with an automatic sampler. The split mode was used for injections. The injection port, ion source, and interface were maintained at 250 °C. Separations were carried out on a fused silica column (12.5 m × 0.20 mm i.d.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness 0.33 μm) (Hewlett Packard). Helium was the carrier gas at an inlet pressure of 40 kPa. Selected-ion monitoring was performed at 70 eV using the electron-impact mode of ionization. Prior to analysis, oligonucleotide samples (~1 nmol) were digested enzymatically with bacterial alkaline phosphatase and snake venom phosphodiesterase and then hydrolyzed with 0.5 mL of formic acid (88%) in evacuated and sealed tubes at 150 °C for 30 min. Samples were then lyophilized and subsequently converted to trimethylsilyl (TMS) derivatives in Teflon-capped hypovials (Pierce) with 0.1 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)/acetonitrile (2:1 v/v) at 130 °C for 30 min. After cooling, the derivatized products were injected directly onto the GC without further treatment.

Restriction enzyme digestions and 0.8% agarose gel electrophoresis were performed as described in Lasko et al. (1987). Denaturing 5% polyacrylamide [19:1 acrylamide:bis(acrylamide)] gel electrophoresis was carried out at 350 V. Single-stranded M13 DNA was sequenced by the method of Sanger et al. (1977) and electrophoresed at 2000 V in 8% polyacrylamide gels containing 7 M urea.

*Synthesis of 8-Hydroxy-2′-deoxyguanosine 5′,3′-Bisphosphate (pd$G^{8-OH}$p).* A modification of the procedure of Kasai and Nishimura (1984) was used to prepare pd$G^{8-OH}$p. The bisphosphate pdGp (20 mg) was dissolved in 5.5 mL of 0.1 M $NaH_2PO_4$ (pH 6.8) containing ascorbic acid (14 mM) and hydrogen peroxide (50 mM) and stirred for 2 h at 25 °C. The major product was purified from the reaction mixture by anion-exchange HPLC (0–0.3 M $KH_2PO_4$ in 30 min at a flow rate of 1.0 mL/min). This $G^{8-OH}$-containing bisphosphate eluted ~3 min before unreacted pdGp. Rechromatography of the collected material showed no evidence of any UV-absorbing impurities. Removal of salt from the product was achieved by passing the collected fractions through a short DEAE-cellulose column (3 × 1 cm) equilibrated in water. The salt was washed from the column with 20 mL of water followed by 20 mL of 0.1 M triethylamine bicarbonate (pH 7.5), and the product was eluted with 0.4 M triethylamine bicarbonate. This material was dried, resuspended in 2 mL of water, and applied to a column packed with Sephadex-SP C-25 (20 × 2.5 cm) (equilibrated in water) to exchange the cation to sodium (Brennan et al., 1983). The UV-absorbing fractions eluting from the column were pooled and dried to give 0.95 mg (4.8%) of the final product, pd$G^{8-OH}$p: UV $\lambda_{max}$ (water) 246, 295 nm [lit. (d$G^{8-OH}$) (Kasai & Nishimura, 1984) 245, 293 nm]. The mass spectrum of the compound was obtained following its hydrolysis in formic acid and derivatization to

[2] Mention of commercial products does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.

the TMS derivative. The two characteristic ions for the molecule at $m/z$ = 455 ($M^+$) and $m/z$ = 440 ($M - CH_3$) (Dizdaroglu, 1985) were observed. Dephosphorylation of $pdG^{8\text{-}OH}p$ with alkaline phosphatase yielded a derivative with identical UV spectral characteristics (at pH 2, 7, and 12) and chromatographic properties to a $dG^{8\text{-}OH}$ standard, prepared as above.

*T4 RNA Ligase Reaction Conditions.* Reaction mixtures were prepared essentially as described in Brennan et al. (1983) and carried out in a volume of 10 μL at 17 °C for 5–7 days. Periodically, 1-μL aliquots were removed and analyzed by anion-exchange HPLC (0.1–1.0 M $KH_2PO_4$ in 5% methanol over 40 min at a flow rate of 1.0 mL/min). Each reaction contained 50 mM Tris-HCl (pH 8.0), 1 mM oligonucleotide acceptor, 5 mM pdNp donor, 0.4 mM ATP, 10 mM $MgCl_2$, 1 mM hexaamminecobalt chloride (HCC), 25% w/v poly(ethylene glycol) 8000 (PEG 8000), 8 mM spermine, 40 mM phosphocreatine, 20 mM DTT, bovine serum albumin (10 μg/mL), creatine phosphokinase (175 units/mL), adenylate kinase (170 units/mL), and 30 μM T4 RNA ligase.

*Preparative Synthesis of Oligonucleotides by RNA Ligase.* The acceptor d(GCTA) (0.1 μmol) was allowed to react with a 5-fold molar excess of either pdGp or $pdG^{8\text{-}OH}p$ in a volume of 0.1 mL under the conditions indicated. The reaction mixtures were incubated for 148 h, at which time the products, d(GCTAG)p and $d(GCTAG^{8\text{-}OH})p$, were purified by anion-exchange HPLC with the elution system used for analytical ligase reactions. The samples were desalted through a Sephadex G-10 column (20 × 2.5 cm). Final yields were estimated by UV spectroscopy assuming approximate molar absorptivity values at 260 nm of 54 900 and 50 500 for the unmodified and adducted products, respectively [$\epsilon_{260}(dG^{8\text{-}OH}) \sim 7300$]. Approximatey 31 nmol of d(GCTAG)p (31%) and 53 nmol (53%) of the adducted pentamer were obtained. These yields probably represent lower limits since various desalting methods were tested before Sephadex G-10 was chosen and some material was lost during these efforts. Approximately 1 $A_{260}$ unit of each pentamer was 3′-dephosphorylated with calf intestinal phosphatase (20 units) by using the protocol of Maniatis et al. (1982). Purification by reversed-phase HPLC and subsequent desalting through Sephadex G-10 afforded the final oligomers, d(GCTAG) and $d(GCTAG^{8\text{-}OH})$.

*Construction of a Bacteriophage M13 Genome Containing a Five-Base Gap in a Unique NheI Restriction Site.* Insertion mutant M13 genomes were prepared by the method of Kunkel (1985). A 47-base oligonucleotide and a 52-base oligonucleotide, complementary to positions 6249–6288 of the M13mp19 genome, but containing either an extra 7 (5′-AAGGCCT-3′) or an extra 12 (5′-AAGGCTAGCCCT-3′) bases between the two cytosine residues in the *Bam*HI restriction site, were used as primers for DNA synthesis with T4 DNA polymerase. A uracil-containing single-stranded M13mp19 DNA was used as the template for both reactions. The smaller of the inserts contained the base sequence coding for the *Stu*I restriction site (5′-AGGCCT-3′), and the larger, the sequence of the *Nhe*I site (5′-GCTAGC-3′). The products of the synthesis reactions were used to transform competent *E. coli* MM294A cells (Basu et al., 1987). Progeny phage were serially diluted and plated in the presence of *E. coli* GW5100, IPTG, and X-Gal (Messing, 1983). Subsequent analysis of the plates revealed that the DNA from both reactions gave rise to plaques of two color types. Plaques from the synthesis involving the 47-base primer were either colorless (82%) or dark blue (18%), while those obtained from the reaction using the 52-base oligomer were either light blue (88%) or dark blue (12%). In each case, the expected insertion mutants gave rise to the more frequently detected plaque colors since the +7 mutant, designated M13+7-(*Stu*I), is a +1 frameshift mutant and results in a nonfunctional lacZ protein and colorless plaques, whereas the +12 mutant [M13+12-(*Nhe*I)] contains an in-frame *amber* (5′-TAG-3′) codon that is partly suppressed in *E. coli* GW5100. Partial suppression leads to the formation of light blue plaques. The dark blue plaques found on both sets of plates presumably arose from a population of the template DNA that escaped uracil glycosylase mediated inactivation of the (+) strand in *E. coli* MM294A. To substantiate these findings, individual plaques were picked (10 putative mutant clones and two probable M13mp19 wild types) from the two sets and used to inoculate *E. coli* GW5100. Following incubation, phage were isolated, and single-stranded DNA was purified. DNA sequencing showed that all of the colorless or light blue plaques examined arose from phage containing the expected inserts. The DNA from the dark blue plaques contained the sequence of wild-type M13mp19. Restriction enzyme digestions were also carried out on replicative form (RF) DNA isolated from cells inoculated with either M13+7-(*Stu*I) or M13+12-(*Nhe*I). As expected, the DNA containing the +7 insert was refractory to cutting by *Bam*HI but linearized with *Stu*I while the DNA with the 12-base insert was resistant to *Bam*HI but sensitive to *Nhe*I.

RF DNA from both mutants was used to create gapped heteroduplexes (GHD) containing a five-base gap in the (+) or (−) strand within the *Nhe*I restriction site. The heating method of Green et al. (1984) was used to anneal 25 μg of *Stu*I linearized M13+7-(*Stu*I) DNA to an equal amount of M13+12-(*Nhe*I), which had been linearized with *Bgl*II and 5′-dephosphorylated with calf intestinal phosphatase (Maniatis et al., 1982). The hybrid molecules produced contained a nonligatable nick in the strand opposed to the five-base gap.

*Phosphorylation of Pentamers and Ligation into GHD.* In separate reactions, 250 ng of either d(GCTAG) and $d(GCTAG^{8\text{-}OH})$ was phosphorylated with [$\gamma$-$^{32}$P]ATP (20 μCi) by polynucleotide kinase (30 units) at 37 °C for 20 min. Unlabeled ATP was added to a concentration of 1 mM, and the incubation was continued for a further 15 min. The reactions were terminated by heating to 65 °C for 15 min, and one-tenth of the reaction volume was removed for HPLC determination of the specific activity of the oligomers. The pentamers were ligated into the GHD (1 μg) with DNA ligase (800 units) by the method described in Lasko et al. (1987). The final reaction volume was 0.1 mL. After 16 h unincorporated pentamers, ATP, and other salts were separated from the radiolabeled DNA by passing the ligation mixture through a Sepharose 4B column (15 × 0.75 cm) preequilibrated with 10 mM Tris-HCl (pH 7.8), 0.1 M NaCl, and 1 mM $Na_2$-EDTA The DNA eluted in the void volume.

*Transformation of E. coli Cells with Site-Specificially Modified Genomes.* Site-specifically modified genomes required for bacterial transformation experiments were prepared essentially as above except that the pentamers were phosphorylated with unlabeled ATP (1 mM) at 37 °C for 30 min. After ligation, the DNA was precipitated, washed twice in 1 mL of 80% ethanol, and resuspended in TE buffer (pH 8.0) (Maniatis et al., 1982). Next, the genomes were incubated with *Stu*I (20 units) to relinearize any RF M13+7-(*Stu*I) DNA that had not hybridized during GHD preparation and had recircularized in the ligation step. The solution was extracted with an equal volume of phenol saturated with Tris-

HCl (pH 8.0), and the DNA was reprecipitated. Immediately before transformation, the DNA was dissolved in 5 μL of water, heated to 100 °C for 3 min to produce single-stranded genomes, and cooled rapidly on ice to inhibit strand renaturation. The denatured DNA was used to transform *E. coli* DL7 by using the process of electroporation. In parallel, 100 mL of LB media (Maniatis et al., 1982) was inoculated with a fresh overnight culture of *E. coli* DL7 (1 mL), and the cells were incubated until they had reached a density of $\sim 1 \times 10^8$ cells/mL. The bacteria were harvested by centrifugation at 5000*g* for 10 min at 4 °C, resuspended in 100 mL of water, and recentrifuged at 5000*g* for 30 min. This procedure was repeated except the cells were resuspended in 40 mL of water. The bacterial pellet was resuspended in 200 μL of glycerol/water (10% v/v) and kept on ice until required. For each transformation 40 μL of the cell suspension was added to the 5-μL DNA solution and then transferred to the bottom of a cold Bio-Rad Gene Pulser cuvette. Cells were electroporated in a Bio-Rad Gene Pulser set at 25 μF and 2.5 kV with the Pulse Controller set at 200 Ω. Following electroporation, the cuvette was removed from the apparatus and 1 mL of SOC medium (Hanahan, 1985) was added immediately to the cell suspension. The cells were then transferred to a 1.5-mL microcentrifuge tube. A portion of the bacteria was plated immediately in the presence of *E. coli* GW5100, IPTG, and X-Gal (Messing, 1983) to determine transformation efficiencies and to be certain that each plaque was independent in origin. The remainder of the cells was incubated for 1.5 h at 37 °C to allow for phage replication and centrifuged at 15000*g* (10 min), and the phage-containing supernatant was stored at 4 °C.

*Sequencing of Mutant M13 DNA.* Seventy-five independent M13 mutants displaying dark blue plaques produced from transfection of site-specifically modified genomes were picked and replated to verify purity. A single plaque from each of the secondary plates was used to prepare single-stranded DNA for sequencing.

## Results

*Synthesis and Characterization of an Oligonucleotide Modified with a Single $G^{8-OH}$ Adduct.* T4 RNA ligase catalyzes the formation of a 3′,5′-phosphodiester bond between a DNA oligonucleotide acceptor (≥3 bases in length) bearing a 3′-hydroxyl group to a donor molecule (which can be as small as a single nucleotide) containing a 5′-phosphate (Brennan et al., 1983). The donor is generally blocked with a 3′-phosphate group to terminate the reaction after a single acceptor/donor condensation.

We chose to use RNA ligase to construct two pentanucleotides, d(GCTAG$^{8-OH}$)p and an unmodified control, d(GCTAG)p. The starting materials for these syntheses were the oligonucleotide acceptor d(GCTA) and either of the two donors, pdG$^{8-OH}$p or pdGp. In order to optimize the reaction conditions needed for these reactions, various analytical reactions (10 nmol of acceptor) were attempted. Product yields from the reactions were monitored by determining the relative decrease in d(GCTA) with respect to the formation of the single peak eluting with a later retention time from an anion-exchange HPLC column (Figure 1). We found that by using a reaction system containing an ATP regeneration system (Brennan et al., 1983) and the DNA and RNA ligase stimulants, PEG 8000 and HCC (Tessier et al., 1986), we were able to obtain very high (>95%) yields of both d(GCTAG$^{8-OH}$)p (Figure 1) and d(GCTAG)p. Under these conditions, both pdGp and pdG$^{8-OH}$p ligated with an equal efficiency.

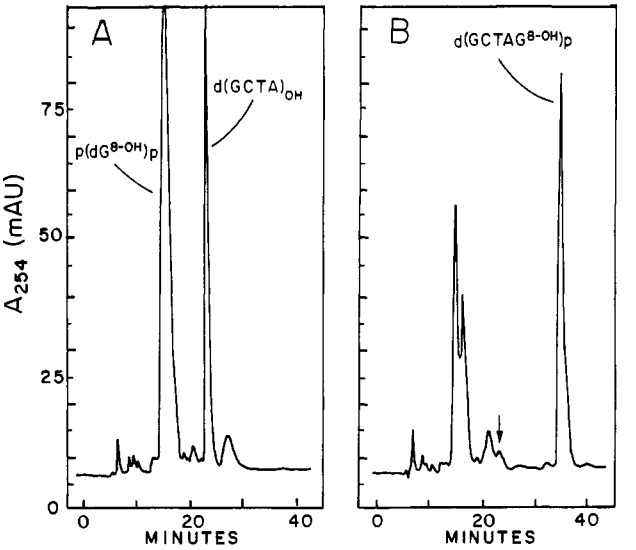


FIGURE 1: Anion-exchange HPLC profiles showing the formation of d(GCTAG$^{8-OH}$)p in a T4 RNA ligase catalyzed reaction between the oligonucleotide acceptor d(GCTA) and the adducted donor pdG$^{8-OH}$p. The reaction was analyzed immediately following addition of the enzyme to the reaction mixture (A) and after incubation at 17 °C for 120 h (B). Chromatographic conditions: 0.1–1.0 M $KH_2PO_4$ in 5% methanol over 40 min at a flow rate of 1.0 mL/min.

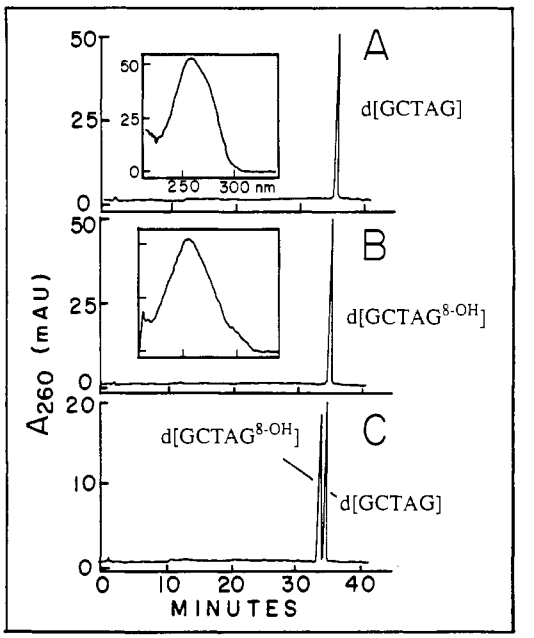


FIGURE 2: Reversed-phase HPLC profiles and UV spectra (insets) of the oligonucleotides d(GCTAG) (A) and d(GCTAG$^{8-OH}$) (B). Ordinate values of insets are in milliabsorbance units. Chromatogram C shows the trace obtained following a coinjection of these two pentamers. Chromatographic conditions: 0–10% acetonitrile in 0.1 M $NH_4OAc$ over 40 min at a flow rate of 1.0 mL/min.

These reactions were repeated on a preparative scale using 0.1 μmol of acceptor. After 148 h the products were collected by HPLC and desalted through Sephadex G-10. In a final step, the pentamers were incubated in the presence of calf intestinal phosphatase to remove the 3′-phosphate group, and the dephosphorylated oligomers were purified by reversed-phase HPLC. Dephosphorylation caused the compounds to elute from the column at a later retention time than their phosphorylated precursors. HPLC profiles of the purified pentamers, d(GCTAG) and d(GCTAG$^{8-OH}$), are shown in Figure 2. The adducted and unadducted species could be resolved although they differ only by the presence of the hy-

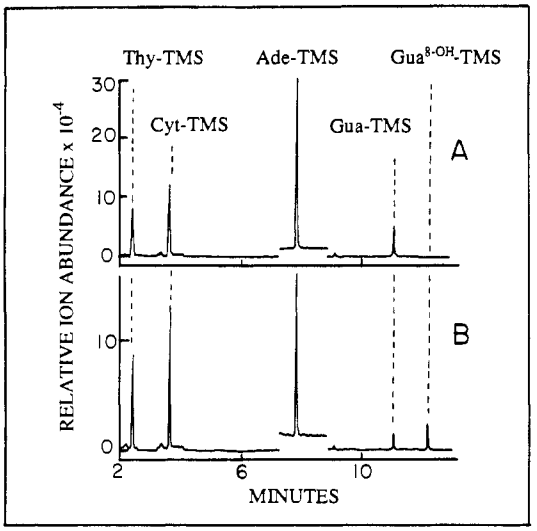


FIGURE 3: Ion-current profiles obtained during GC–MS/selected-ion monitoring analysis of TMS-derivatized hydrolysates of oligonucleotides. (A) d(GCTAG); (B) d(GCTAG$^{8-OH}$). Derivatized bases were monitored simultaneously at $m/z$ = 255 (thymine), 240 (cytosine), 264 (adenine), 352 (guanine), and 440 (G$^{8-OH}$).

droxyl group (Figure 2C). Good evidence that the modified pentamer contained G$^{8-OH}$ was obtained by comparing its UV spectrum to that of the unadducted control (insets, panels A and B of Figure 2). These data showed that the spectrum of the adducted ligation product, d(GCTAG$^{8-OH}$), contained an extra UV chromophore centered at approximately 300 nm. The presence of an 8-hydroxylated guanine residue in the pentamer would be expected to have this effect since the pdG$^{8-OH}$p monomer has an absorbance maximum at 295 nm. However, to obtain more definitive proof that this compound contained an intact G$^{8-OH}$ moiety, both control and modified compounds were analyzed by GC–MS following enzymatic digestion, hydrolysis in formic acid, and base derivatization with BSTFA. The presence of the four normal bases as well as G$^{8-OH}$ in the molecules was assessed by analyzing the compounds entering the mass spectrometer from the gas chromatograph by selected-ion monitoring at $m/z$ values characteristic for the TMS-derivatized bases ($m/z$ = 440, G$^{8-OH}$; 255, thymine; 240, cytosine; 264, adenine; 352, guanine). The resultant ion chromatograms (Figure 3) showed that the adducted compound contained significant amounts of a compound that had a GC retention time and molecular ion mass identical with those of an authentic sample of TMS-derivatized G$^{8-OH}$, whereas the control pentamer contained only the four unmodified bases.

Quantitative assessment of the base composition of the oligonucleotides was carried out after digestion of the compounds to their constituent nucleosides with bacterial alkaline phosphatase and snake venom phosphodiesterase and subsequent analysis by HPLC. A chemically synthesized control, d(GCTAG), was analyzed in parallel. The chromatogram obtained following digestion of d(GCTAG$^{8-OH}$) is shown in Figure 4, and a summary of the data is presented in Table I. These results indicated that the unmodified pentamer had a base composition identical with that of the chemically synthesized standard (Table I, columns 2 and 4). As expected, the adducted product contains only half as much deoxyguanosine as the other two compounds and a stoichiometric amount of material identical by HPLC and UV spectrum with authentic dG$^{8-OH}$ (Table I, column 3).

*Construction of an M13 Vector To Study G$^{8-OH}$ Mutagenesis.* The aim of this aspect of the work was to determine

Table I: Relative Nucleoside Content of Two Oligonucleotides, d(GCTAG$^{8-OH}$) and d(GCTAG), Synthesized by a Combination of Chemical and T4 RNA Ligase Mediated DNA Synthesis[a]

| | relative nucleoside content | | |
|---|---|---|---|
| | d(GCTAG)[b] | d(GCTAG$^{8-OH}$)[b] | d(GCTAG)[c] |
| dC | 1.0 (1.0)[d] | 1.0 (1.0) | 1.0 (1.0) |
| dG | 3.07 (3.20) | 1.53 (1.60) | 2.97 (3.20) |
| dT | 1.20 (1.19) | 1.18 (1.19) | 1.18 (1.19) |
| dA | 1.90 (2.07) | 1.86 (2.07) | 1.86 (2.07) |
| dG$^{8-OH}$ | nd[e] | 0.92 (0.97) | nd |

[a] Relative content of constituent nucleosides was calculated by dividing the integrated HPLC peak area for each nucleoside by the relevant extinction coefficient at 260 nm and normalizing to dC. [b] Prepared with RNA ligase. [c] Chemically synthesized standard. [d] Theoretically expected peak area ratios (relative to dC) shown in parentheses. [e] nd, not detected.

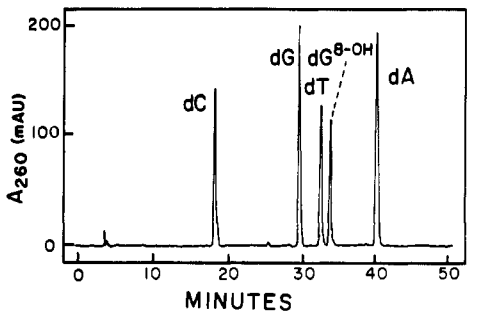


FIGURE 4: Reversed-phase HPLC chromatogram obtained following bacterial alkaline phosphatase/snake venom phosphodiesterase digestion of d(GCTAG$^{8-OH}$). Chromatographic conditions: 0–7.5% acetonitrile in 0.1 M $NH_4OAc$ over 50 min at a flow rate of 0.75 mL/min.

the genetic effects of a single G$^{8-OH}$ adduct in *E. coli*. Our approach was to construct an M13 vector suitable for ligation of the adducted and unadducted pentamers into a unique *Nhe*I (5′-GCTAGC-3′) restriction site, where the underscored bases are part of an in-frame *amber* codon. A key feature of this system is that it provides two independent methods of mutant selection during the in vivo studies. First, since the adduct resides in the guanine residue of the nonsense codon (which causes the formation of light blue plaques on *Sup*E hosts), the majority of plaques resulting from targeted misprocessing of the adduct (base substitutions) will be dark blue. Second, mutant DNA will also be insensitive to cutting by *Nhe*I, a property that permits the enrichment of phage containing any targeted mutation. To create GHD suitable for this project, two M13 vectors were constructed. One of these, M13+7-(*Stu*I), contains a *Stu*I restriction site and an additional 3′ thymine residue (5′-AGGCCTT-3′) within the (+) strand sequence of the *Bam*HI site in M13mp19. This vector gives rise to colorless plaques after bacterial transformation. The second construct contains the *Nhe*I restriction site and has the base sequence 5′-AGGGCTAGCCTT-3′ within the *Bam*HI site. This vector, M13+12-(*Nhe*I), possesses the in-frame *amber* codon. The method used for making GHD containing a five-base gap in either the (+) or (−) strand from these genomes is outlined in Figure 5. Since the pentamers have a non-self-complementary, 5′-GCTAG-3′, base sequence, oligonucleotide ligation is limited to the GHD containing the five-base gap in the (+) strand.

Ligation of the oligonucleotides d(GCTAG) and d(GCTAG$^{8-OH}$) into the GHD was accomplished by using DNA ligase after 5′-phosphorylation of the pentamers with [$\gamma$-$^{32}$P]ATP (Figure 5). A partial restriction map of the adducted ligation product is shown in Figure 6. Immediately after ligation and purification, a portion of the radiolabeled

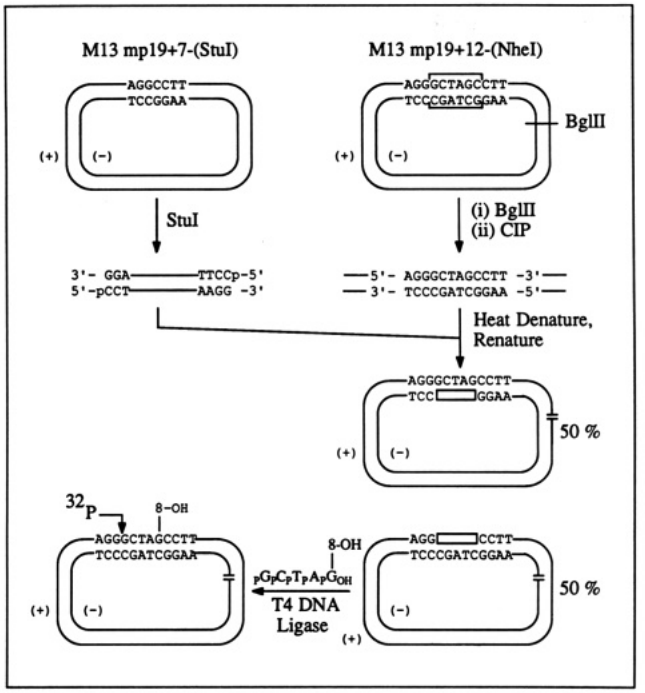


FIGURE 5: Protocol for preparation of a site-specifically modified M13mp19-derived genome containing a single $G^{8-OH}$ adduct at position 6276. The pentamer d(GCTAG$^{8-OH}$) was ligated into a *Nhe*I restriction site within the GHD, which were prepared by annealing *Stu*I-linearized M13+7-(*Stu*I) DNA with *Bgl*II-linearized, 5′-dephosphorylated, M13+12-(*Nhe*I) DNA. See text for details. CIP is calf intestinal phosphatase.

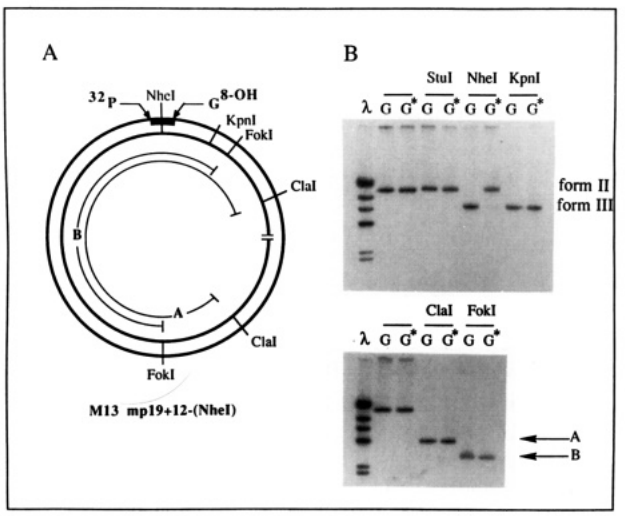


FIGURE 6: Frame A: Partial restriction map of the genome obtained from ligation of [$\gamma$-$^{32}$P]ATP-phosphorylated d(GCTAG$^{8-OH}$) into the M13mp19-derived GHD. Frame B: Autoradiogram of an 0.8% agarose gel to show migration of radiolabeled DNA fragments obtained from the adducted (lanes headed G*) and unmodified (lanes headed G) ligation products, and after incubation of the DNA with the restriction enzymes *Stu*I, *Nhe*I, *Kpn*I, *Cla*I, or *Fok*I. The *Hin*dIII-digested λ DNA size markers are shown in the left-hand lanes.

DNA was electrophoresed through an 0.8% agarose gel and autoradiographed (Figure 6). The product was radioactive DNA that comigrated with a nicked-circular (form II) DNA standard. We were able to calculate the efficiency of ligation by correlating the specific activity of the labeled oligonucleotides (determined by HPLC) with the amount of radioactivity incorporated into form II DNA. These calculations gave an approximate efficiency of 25% (50% of the theoretical maximum) for the control ligation and 23% for the ligation of d(GCTAG$^{8-OH}$) (46% of the theoretical maximum).

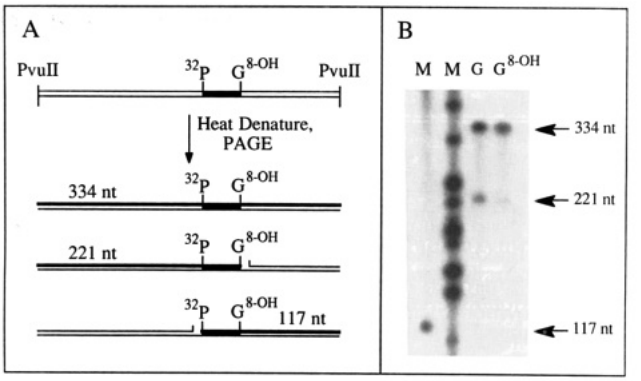


FIGURE 7: Determination of the extent of oligonucleotide ligation in $G^{8-OH}$-adducted and unmodified M13mp19-derived genomes. Frame A: Map of the 334-nt *Pvu*II restriction fragment containing the ligated pentamers. Bold lines indicate the expected radioactive single-stranded DNA fragments obtained following *Pvu*II digestion, heat denaturation, and electrophoresis through a denaturing gel. Complete 5′ and 3′ ligation of the pentamers into the GHD will yield a 334-nt fragment whereas one-sided ligation at either the 5′ or 3′ side produces $^{32}$P-labeled fragments of 221 or 117 nt, respectively. Frame B: Autoradiogram of resultant gel showing the radioactive fragments obtained from either the control (lane headed G) and adducted (lane headed $G^{8-OH}$) vectors. The lanes headed "M" are single-stranded DNA size markers.

Incorporation of the $^{32}$P label into the genomes also allowed us to demonstrate that the oligonucleotides had been ligated into the correct position in the GHD. Restriction mapping of the DNA with the enzymes *Stu*I, *Nhe*I, *Kpn*I, *Cla*I, and *Fok*I was used to show that the radiolabel comigrated with the expected fragments after digestion and agarose gel electrophoresis (Figure 6). As expected, both vectors were insensitive to cleavage by *Stu*I. However, although the control DNA was linearized by *Nhe*I, the adducted genome was refractory to cutting (>95%) by a 200-fold excess of this enzyme (Figure 6). This finding is consistent with the presence of a structural deformation, such as a DNA adduct, within the restriction site inhibiting the enzyme. We have observed similar effects when other DNA adducts including $O^6$-methylguanine (Green et al., 1984) and the C8-guanine adduct of 4-aminobiphenyl (Lasko et al., 1987) are built into restriction enzyme recognition sequences.

These experiments show that both d(GCTAG) and d(GCTAG$^{8-OH}$) can be covalently ligated into a five-base gap within M13mp19-derived GHD. The data do not indicate, however, what percentage of ligation events occurred at both 5′ and 3′ termini. To determine this, control and adducted DNAs were digested with *Pvu*II, and the products were separated through a 5% denaturing polyacrylamide gel (Naser et al., 1988). Theoretically, three radioactive fragments could be formed (see Figure 7A) depending on whether ligation occurred at both ends of the pentamer (334 nt), or at the 5′ (221 nt) or 3′ (117 nt) ends only. Only bands corresponding to 334 and 221 nt fragments were observed (Figure 7B) from either DNA after autoradiography of the gel. Densitometric studies indicated that ~70% of all ligations had occurred at both termini during ligation of the control oligomer. Surprisingly, we observed a greater percentage of complete ligation with the pentamer containing the $G^{8-OH}$ adduct. Here, approximately 90% of the radioactivity comigrated with the 334-nt fragment.

*Genotoxicity of a Single $G^{8-OH}$ Adduct in Single-Stranded M13 DNA following Transformation of E. coli Cells.* The ultimate goal of these studies was to determine the genetic effects of $G^{8-OH}$ after transfection of the single-stranded site-specifically modified genome into *E. coli*. Following li-

Table II: Survival and Mutation Frequency of $G^{8\text{-OH}}$-Modified Single-Stranded M13 DNA in *E. coli*

| expt no. | no. of infective centers | | mutation frequency (all mutations) (%)[d] | | mutation frequency (G → T transversions) (%)[e] | |
|---|---|---|---|---|---|---|
| | G[b] | $G^{8\text{-OH}}$[c] | G | $G^{8\text{-OH}}$ | G | $G^{8\text{-OH}}$ |
| 1 | $8.18 \times 10^6$ (100)[a] | $3.96 \times 10^6$ (48) | 0.15 | 1.20 | nd[f] (0/8) | 0.5 (7/18) |
| 2 | $7.32 \times 10^6$ (100) | $6.66 \times 10^6$ (91) | 0.10 | 1.35 | nd (0/7) | 0.5 (6/16) |
| 3 | $6.99 \times 10^6$ (100) | $5.60 \times 10^6$ (80) | 0.20 | 2.35 | nd (0/8) | 1.0 (8/18) |

[a] Relative values shown in parentheses. [b] Control genome. [c] Adducted genome. [d] Defined as number of dark blue plaques/total plaques. [e] Determined by multiplying the fraction of dark blue plaques containing a G → T transversion (in parentheses) by the frequency of all mutations giving a dark blue plaque phenotype. [f] nd, not detected.

gation of nonradiolabeled phosphorylated pentamers into the GHD (~1.8 μg), the adducted and unadducted genomes were incubated with *Stu*I to relinearize any M13+7-(*Stu*I) RF DNA that had recircularized during ligation. This step was essential since RF DNA would be transfected more efficiently than the single-stranded genomes obtained upon heat-induced denaturation of the required ligation products and so could have masked any small effects that $G^{8\text{-OH}}$ may have on M13 viability. Immediately before transfection the DNA was heated to 100 °C to prepare single-stranded genomes and cooled on ice. Transformation efficiency data obtained from these experiments were calculated from the numbers of infective centers obtained from plating a portion of the transformation mixture immediately after electroporation and addition of SOC medium. The data shown in Table II indicate that, in each of the three independent experiments carried out, the adducted genome consistently gave rise to a lower number of infective centers than the control. Although small and varied in magnitude, this decrease in survival (ranging from 9 to 52%) could suggest that a fraction of the $G^{8\text{-OH}}$ adducts inhibited DNA replication. Alternatively, the lesion may have been lost from the genome as the result of a chemical and/or enzymatic process, and this eliminated the viability of the single-stranded vector.

The relative proportions of each of the three possible plaque types (dark blue, light blue, or colorless) obtained from the transformations were determined. Theoretically, all of the dark blue and colorless plaques should have resulted from mutation of the site-specifically modified genomes. This was unlikely, however, because even though a *Stu*I digestion procedure was used to reduce the viability of any parental RF M13+7-(*Stu*I) DNA prior to transformation (see above), a small proportion of colorless plaques probably still resulted from successful transfection of this vector. Consequently, only dark blue plaques were scored directly as mutants. The data presented in Table II show that the adducted genomes gave rise to a higher percentage of dark blue plaques (~1.6%) than the control DNA (~0.2%) (average of three experiments). To investigate what types of mutations were responsible for this plaque color, 75 independent dark blue plaques were isolated and used to prepare single-stranded DNA for sequencing.

Sequence data from DNA derived from the adducted genome indicated that 38–44% of mutants that had given rise to a dark blue plaque contained a G → T transversion mutation at position 6276 [(+) strand] in the *Nhe*I restriction site (GCTAGC → GCTATC) over the three separate experiments (Table II). Overall, the frequency of this transversion is ~0.7%. It is highly probable that this mutation occurred as a consequence of the $G^{8\text{-OH}}$ adduct since the lesion was incorporated at this site. Moreover, this mutation was not seen in mutant DNA sequences derived from the unmodified genome (Table II). Neither the frequency of the G → T transversion nor the total mutation frequency was enhanced by the expression of SOS functions (data not shown); this was established by preirradiating the host for the adducted genome with UV light (50 J/m$^2$) as described (Lasko et al., 1988). The adducted genome also gave rise to two nontargeted double base pair substitutions. The frequencies of these mutations, GCTAGC → GCACGC and GCTAGC → GCATGC, were very low (<0.1%), and although neither was detected in the control experiment, this may have been because too few of the unmodified genome-derived mutants were analyzed for these sequence changes to be observed.

The remaining 60% of the mutations that yielded a dark blue plaque phenotype in phage DNA arising from the adducted genome were common to the mutations found in the control experiment. These consisted of (i) a large in-frame deletion (66 base pairs) including the *Nhe*I site, (ii) various scrambled sequences adjacent to the ligation site, or (iii) a T → C transition or a T → A transversion at position 6274 [(+) strand], an A → G transition or an A → C transversion at position 6275 [(+) strand], or a G → C transversion at position 6276 [(+) strand]. These base substitutions, positioned in the *amber* codon, represented 70% of the mutations induced by the unmodified genome and a similar proportion (80%) of the mutations [types i–iii] obtained from transfection of the adducted vector. We presume that the first two types of mutations occurred as a consequence of the genetic engineering steps used to prepare the genomes. The basis of the base pair substitutions (type iii) is less clear, although very low levels of impurities in the two pentamers d(GCTAG) and d(GCTAG$^{8\text{-OH}}$) could have engendered this class of mutations. We note, however, that during the course of the work we synthesized several independent preparations of oligonucleotides and detected no batch-dependent variations in the types or amounts of mutations arising following ligation and transformation.

## Discussion

Site-specifically modified DNA oligonucleotides are convenient models for probing the chemical and biological effects of individual carcinogen adducts (Basu & Essigmann, 1988). Generally, the favored method of preparation of these molecules is by chemical synthesis. There is a need to develop gentler methods of synthesis, however, as evidenced by the large number of DNA adducts that are unstable to the harsh conditions of both chemical synthesis and subsequent deprotection. Recently, Preston and Loeb (1988) reviewed the advantages and disadvantages of various enzymes capable of mediating the synthesis of site-specifically modified oligonucleotides. Of these, T4 RNA ligase is possibly the best suited to general application with a wide range of DNA adducts. The enzyme has already been used to synthesize a variety of DNA or RNA oligonucleotides containing modified bases (Preston & Loeb, 1988). RNA ligase reactions also take place at a pH (7.9–8.0) and temperature (17–25 °C) favorable for syntheses involving adducts that are labile to extremes of acidity, alkalinity, and temperature. Although the reactions with DNA precursors are slow (up to 10 days), high yields can be obtained, as evidenced here. Importantly, the conditions required for constructing nanomole quantities of DNA oligonucleotides are known in detail (Brennan et al., 1983; Tessier et al., 1986).

We have optimized RNA ligase reaction conditions to enable efficient ligation (>95%) of an pdG$^{8-OH}$p or an unadducted pdGp monomer onto the tetranucleotide d(GCTA). To achieve high yield, two stimulants of DNA and RNA ligases, HCC and PEG 8000, were required in the reaction buffer (Tessier et al., 1986). Both compounds are able to increase the local concentration of reactants either by size exclusion (PEG 8000) or by increasing mutual ionic interactions (HCC) (Pheiffer & Zimmerman, 1983; Harrison & Zimmerman, 1984; Rusche & Howard-Flanders, 1985). Although the absolute yields of the pentanucleotides were small by comparison with the amounts obtainable by total chemical synthesis, we were able to produce amounts of oligonucleotide sufficient for characterization using a combination of chromatographic and spectroscopic techniques. Present studies being carried out in our laboratories indicate that the RNA ligase reaction conditions used here should be suitable for a range of carcinogen adducts unstable to chemical synthesis.

Transformation of wild-type *E. coli* cells with the site-specifically adducted genomes demonstrated that the G$^{8-OH}$ adduct induced a targeted G → T transversion mutation in progeny phage DNA at a frequency of ~0.7%. Although seemingly modest in magnitude, we consider this value to be reasonable and significant in light of data from in vivo site-specific mutagenicity studies with other adducts such as $O^6$-methylguanine (Loechler et al., 1984). The mutation frequency (G → A transition) of $O^6$-methylguanine was only 0.4% when a single-stranded M13mp8 genome containing the lesion was used to transform *E. coli* MM294A cells. The mutation frequency was elevated, however, to values approaching 20% by treating the competent bacteria with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine prior to transformation. This alkylating agent had the effect of depleting the intracellular stores of the $O^6$-methylguanine repair proteins and thus rendered the cell more sensitive to the mutagenic effects of the site-specific adduct. Similarly, we would anticipate an increase in the inherent mutagenicity of G$^{8-OH}$ by using bacterial strains that are compromised in their ability to repair this adduct. It is important to note that our experiments were performed with single-stranded DNA and most DNA repair enzymes identified to date (with the exception of uracil-DNA glycosylase) require or exhibit a strong preference for duplex DNA (Wallace, 1988; Myles & Sancar, 1989). It is conceivable, however, that if the G$^{8-OH}$-adducted genome is successfully replicated in the first round of replication, DNA repair may play an important role in modulating mutagenesis in subsequent rounds of RF replication.

The mutation analysis presented in Table II was based on the ability of G$^{8-OH}$-induced mutations to cause a plaque color change from light to dark blue. Consequently, it could be inferred that the data may represent only a partial spectrum of the entire range of mutations caused by the adduct. In a preliminary experiment (data not shown), a more exhaustive search for all mutations was made by enriching for mutants that produce either light blue or colorless plaques, after removal of the parental M13+12-(*Nhe*I) DNA and any remaining M13+7-(*Stu*I) DNA by serial rounds of *Nhe*I and *Stu*I digestion (Loechler et al., 1984). This single experiment showed no evidence of G$^{8-OH}$-induced mutations other than the G → T transversion at a limit of detection of ~0.1%. Hence, these preliminary findings, when taken together with the data of Table II, indicate that the major mutagenic event induced by G$^{8-OH}$ in vivo is the G → T transversion.

The diversity of the adduct population following the exposure of DNA to oxidants or ionizing radiation is likely to be the cause of the many different types of mutations induced in genomes of mammalian (Grosovsky et al., 1988), bacterial (Glickman et al., 1980), and viral origin (Ayaki et al., 1986; Tindall et al., 1988; Hoebee et al., 1988; Moraes et al., 1989). The mutational spectra observed in these assay systems show a preference for single base changes, especially base pair substitutions, the majority of which tend to be transitions or transversions at G·C pairs (Glickman et al., 1980; Ayaki et al., 1986; Tindall et al., 1988; Hoebee et al., 1988; Hayes et al., 1988; Moraes et al., 1989). The likely mechanism for the most frequent event, the G·C → A·T transition, involves the deamination of an unstable oxidation product of cytosine (Hayes et al., 1988). The resulting modified uracil could form a base pair with adenine during DNA replication, and this event would give rise to the C → T transition. This mechanism, however, would not account for the occurrence of G·C → T·A and G·C → C·G transversions, which are also observed as significant features of the mutational spectrum. For these mutations other pathways must be involved. By using site-specific mutagenesis, we have provided direct evidence that G$^{8-OH}$ is a premutagenic adduct in vivo and, more specifically, that it could contribute to the G·C → T·A transversions known to be induced by ionizing radiation and chemical oxidants.

The targeted G → T mutation we observed was only one of the many mutations seen for G$^{8-OH}$ in vitro (Kuchino et al., 1987), where the lesion frequently induced a variety of base substitution mutations at the site of the adduct and also at the adjacent 5′ and 3′ bases. One possible reason for the differences between the in vivo and in vitro data is that G$^{8-OH}$ is a likely substrate in vivo for repair enzyme(s) that remove it from the genome before it can be encountered by the replication machinery of the cell. This explanation could be expanded to include the mechanism underlying the G → T transversion mutations seen in our experiments if the adducted base were repaired by an enzyme that left an AP site in the DNA (e.g., by a DNA glycosylase). AP sites are mutagenic in vivo (Loeb & Preston, 1986), and the predominant base change observed is a result of the insertion of dATP opposite the lesion.

An alternative pathway leading to the G$^{8-OH}$-induced G → T mutations may reflect structural and conformational changes imposed by the adducted purine within the DNA helix. Hydroxylation of the C8 position of guanine, in common with substitution at this position by some other electrophiles [e.g., a variety of aromatic amines (Swenson & Kadlubar, 1981)], is believed to induce the base to assume a syn conformation (Kuchino et al., 1987). Furthermore, the modified guanine moiety exists predominantly in the 6,8-diketo tautomeric form (Culp et al., 1989). Reorientation of guanine to a syn conformation in DNA forces the exocyclic $N^2$ group out of the minor groove and removes its ability to participate in base-pairing interactions (Topal & Fresco, 1976; Loechler, 1989). Conversely, the N7 atom of a *syn*-guanine assumes a position in the base-pairing region of the helix. The N7 group would likely be protonated in the keto tautomer of G$^{8-OH}$ and, together with the hydrogen bond accepting $O^6$ group, could form a G·A mispair during replication. This would lead to the G·C → T·A transversion mutations in our experiments (Loechler, 1989).

We are currently investigating the genetic requirements for G$^{8-OH}$ mutagenesis by using *E. coli* strains with different DNA replication and repair backgrounds. The data obtained from these experiments should provide further details on the mechanisms underlying the type of mutation(s) induced by the adduct in vivo as well as the relative contribution of G$^{8-OH}$

to the overall spectrum of radiation- and oxidant-induced mutation.

## Added In Proof

A. Grollman and M. Moriya (personal communication) recently have informed us that they have observed the same mutation frequency and essentially the same mutational specificity for $G^{8\text{-}OH}$ as reported here.

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